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Asparaginase enzyme reduces acrylamide levels in fried and wood oven baked pizza base

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ABSTRACT

The possibility to reduce the acrylamide (AA) formation during cooking by treating pizza dough with L-Asparaginase enzyme was explored. Wheat flour was added with commercial enzyme Preventase® in three formulations: W, M and XR-BG during mixing to produce pizza doughs. AA content was evaluated in the obtained pizza bases through UHPLC-UV analysis. Enzymes concentration (0.15 ÷ 12 U/100 g of flour) and water content (58 ÷ 64 g/100 g of flour) during mixing were considered for pizza doughs intended for frying. In the fried pizza base, the untreated sample (without Preventase®) showed an AA level of 3150±554 μg/kg$_{dw}$. Preventase® treatment was able to reduce AA up to non-detectable levels (M and W) or 89% (XR-BG). The enzymatic treatment up to 3–6 U/100 g did not affect the dough handling. Preventase® W (1.5 ÷ 6.0 U/100 g of flour), which showed the best performance in fried pizza samples, with a lower amount of enzyme and optimal dough consistency, was applied in the preparation of the wood oven baked pizza base. In these experiments, the AA level of the untreated pizza base sample was 2210±205 μg/kg$_{dw}$, and the enzymatic treatment resulted in a maximum AA reduction of approximately 50%, without apparently altering the technological characteristics of the dough. Results showed that the use of L-Asparaginase could possibly play a key role in mitigating acrylamide formation in the cooking process of fried and wood oven baked pizza base.

1. Introduction

Acrylamide (AA) is a toxic substance, carcinogenic and dangerous to human health defined a chemical hazard for the food chain by EFSA due to the its presence in cooked food (EFSA, 2015:EN-817). AA is a low molecular weight, highly water soluble, organic compound which forms from the naturally occurring constituents asparagine (Asn) and sugars in certain foods when prepared at temperatures typically higher than 120 °C and low moisture during the Maillard reaction (Mottram et al., 2002; Stadler et al., 2002). Maillard reaction and caramelization are the most important chemical events occurring during the manufacture of bakery and fried cereal products. The World Health Organization (WHO) considers 0.5 μg/L the maximum level for AA in water, however foods such as french fries, baked potato chips, crisp breads were found to contain AA between 50 and 1000 μg/kg (WHO, 2011). The benchmark for EU is 50 μg/kg for wheat based bread and 100 μg/kg for soft bread other than wheat based bread (EU Reg, 2017/2158). In 2011 a Joint FAO/WHO expert committee on food additives, based on dose-response modeling data to evaluate the exposure-related effects of AA in laboratory animals, established a maximum daily consumption limit of 180 μg/kg body weight (b.w.), on the risk of cancer development (WHO, 2011). In European Countries, EFSA lowered the threshold to 170 μg/kg b.w. per day, and clarified that this is not a "recommended value", but a reference point to calculate the margin of exposure (MOE) (EFSA, 2015:EN-817). However, both the authorities recommend to devote great effort into implementation and development of mitigation methods for acrylamide in foods in order to reduce the levels of this substance as much as possible, at least in foods of major importance for dietary exposure, because of its probable carcinogenic effects (WHO, 2011; EU Reg, 2017/2158). Thus, nowadays, the scientific world focuses on mitigation strategies for this substance in cooked foods, especially fried and starchy foods such as cereals (Jia et al., 2021; Sarion et al., 2021). Pizza is a tasty and appetizing food, widespread and consumed all over the world. Its dough is made up of simple ingredients: flour (generally wheat flour), water, salt and yeast. Wheat flour is a strongly starchy matrix and contains Asn-in a wide range of 7.4 to 66.4 mg/100 g (Claus et al., 2006; Stockmann et al., 2018). For these characteristics, pizza doughs are optimal candidates to develop AA dur-

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ing cooking. Furthermore, in the Neapolitan tradition, pizzas are eaten both fried and baked in a wood oven, two high-temperature cooking methods that favor the formation of AA. Therefore, pizzas fall into the product category of major importance for dietary exposure.

One of the measures applied by food business operators to reduce AA in food is the use of the asparaginase enzyme as suggested by the European Commission Regulation in 2017. L-Asparaginase catalyzes the conversion of the Asn, which is the reaction substrate, to aspartic acid and ammonia, preventing the formation of acrylamide (Rottmann et al., 2021). The enzymatic activity of asparaginases is optimal in applications with a water content of 30% at least where good enzymatic activity can be reached. Enzymatic action occurs in two steps: (1) the enzyme needs to find its substrates, usually water and another substrate such as asparagine and convert them into other products; (2) the enzyme needs to release these converted products so that it is ready to start the conversion of more substrates (Xu et al., 2016). Furthermore, the enzyme activity is influenced by the contact with the substrate, which in turn might be affected by the food matrix composition and structure (Anese et al., 2011). With regard to the chemical composition, sufficiently high water contents may favor mobility of the enzyme towards the substrate, promoting hydrolysis of asparagine and thus acrylamide mitigation (Amrein et al., 2004; Hendriksen et al., 2009). From these considerations, it becomes clear, that the presence of substrate, the water content and the mixing regime are the three factors that determine the speed of the enzymatic process. For bakery products, such as bread or biscuits, this incubation time can easily be included in the proofing step (Xu et al., 2016).

Asparaginase effect on the acrylamide formation was investigated by Capuano et al. (2009) that reduced AA formation up to 88% in toasted bread. Kumar et al. (2014) also studied the reduction of acrylamide formation in bakery products, such as sweet bread, by enzyme treatment. With increase in L-Asparaginase level, the acrylamide formation was reduced upon 97% and 73% reduction of acrylamide formation in the crust and crumb regions of bread, respectively. Matouri and Alemzadeh (2018), confirmed the benefit of asparaginases in the subsequent baking process at higher temperatures by analyzing decreased AA levels compared to dough without an enzyme treatment. More recently, the use of a 1% asparaginase solution, namely Preventase® L, decreased the acrylamide concentration by 59% in fried potato (Rottmann et al., 2021). However, there is a lack of studies regarding the levels of AA in pizza in the literature. In this study, AA content in pizza bases was evaluated. Moreover, the use of Preventase®, asparaginase enzyme for food sector, was investigated for reducing AA content in pizza base both fried and wood oven baked. Preventase® W, M or XR-BG were applied at increasing concentrations in pizza doughs prepared with standard recipe and with different percentage of water. The resulting acrylamide levels were evaluated in the fried pizza bases and compared to the untreated control. The best performing enzyme (Preventase® W) was subsequently used for pizza bases cooked in a wood oven. Finally, a discussion was addressed on the prediction of AA intake levels related to the consumption of a fried or wood oven baked pizza.

2. Materials and methods

2.1. Chemicals

AA standard (electrophoresis >99%) was purchased from Biorad company. Methanol, Water (HPLC-grade), Hexane mixture of isomers were purchased from Carlo Erba. All other chemicals and solvents used were of analytical grade and were procured from standard chemical companies. Ultra-pure water (Milli-Q system, USA) was used throughout the experiments. The reverse-phase C18 column used for acrylamide determination is the Nucleodur C18 Gravity 150×3 mm 3 µm and cellulose acetate filters with porosity 0.45 and 0.20 µm were purchased from Macherey-Nagel.

2.2. L-Asparaginase Enzyme

L-Asparaginase enzyme (L-asparagine amidohydrolases EC 3.5.1.1) produced from *Aspergillus niger*, was generously donated by the DSM company (NL) in 3 different formulations in pale white powder form: Preventase® W (containing wheat flour), M and XR-BG (containing maltodextrins). These enzymatic preparations have been specially developed for use in the main food industry applications by DMS Company. One Unit of enzymatic activity (U) was defined as the amount of asparaginase that catalyzes the release of one mmol of ammonia from L-asparagine per minute under standard conditions (37 °C/pH=5.0). The specific activity of the three commercial asparaginase preparations was calculates to be as follow: Preventase® *W* = 1.55 U/g, Preventase® *M* = 1.89 U/g, Preventase® XR-BG = 1.71 U/g. Further details are available in Supplementary materials section S1. The enzyme was added to the flour in substitution of the same amount of flour, in order to maintain the overall quantity of solids in the recipe. In the first experiments, the three different enzyme preparations were tested, then Preventase® W was chosen for the following experiments.

2.3. Wheat flour and pizza dough ingredients

Commercial refined wheat flours, Casillo (12% moisture, 0.5% ash, 10% proteins, 73.5% carbohydrate, 2% fiber, 2% lipids) and Caputo (12% moisture, 0.5% ash, 13% proteins, 70% carbohydrate, 3% fiber, 1.5% lipids), were used for the experiments. Caputo dry yeast (freeze-dried brewer's yeast *Saccharomyces Cerevisiae*, containing sorbitan monostearate, E491, as emulsifier; cell viability on PDA medium > 10 log cfu/g), water and salt (common food-grade sodium chloride) were purchased in a local supermarket (Portici, Italy).

2.4. Farinograph analysis

The rheological characteristics of wheat flour as it is or in the presence of different amounts of L-Asparaginase enzyme (Preventase® W, M, or XR-BG) were determined using Brabender farinograph (Brabender® GmbH & Co KG, Duisburg, Germany), fitted with 50 g mixing bowl, according to AACC (1999) methods. The Brabender Units value (BU) was determined at different% of water and the results were expressed as the average value of three replicates for each sample.

2.5. Preparation of fried and wood oven baked pizza bases

Several trials of pizza doughs in triplicate were performed with L-Asparaginase. For fried pizza bases, Casillo flour (250 g), added with each of the three different enzyme preparations (Preventase® W, M and XR-BG) at 0 (control, ctr), 0.15, 0.30, 1.5, 3.0, 6.0, 9.0, 12.0 U/100 g of flour, was mixed with the other ingredients (58% water, 1% salt, 1% dry yeast based on the flour weight) inside a Farinograph-E bowl (300 g) (Brabender® GmbH & Co KG, Duisburg, Germany) and kneaded with speed of 63 min^{-1} at 26 °C until the dough development time occurred (which is defined as the time required to achieve the peak value of BU in the farinogram graph) and for as long as the curve on the farinogram remains constant, indicating the maximum consistency and stability of the dough, before the structure breakdown (15 min). In parallel experiments, other doughs were prepared in the same conditions with increasing amount of water (60, 62 and 64 g/100 g of flour) and 3 U/100 g of Preventase®. The obtained untreated and enzyme-treated doughs were fermented in a leavening chamber maintained at 30 °C and 80% of relative humidity (RH) for 2 h, and then rolled out to form a circular-shaped disk, and cooked by frying in sunflower oil at 190–200 °C for 1 min. The image of a fried pizza base is shown in Figure S2.1 in supplementary material section S2.

For the wood oven baked pizza base, Caputo flour was added with Preventase® W (0, 1.5, 3.0, 6.0 U/100 g of flour) and then

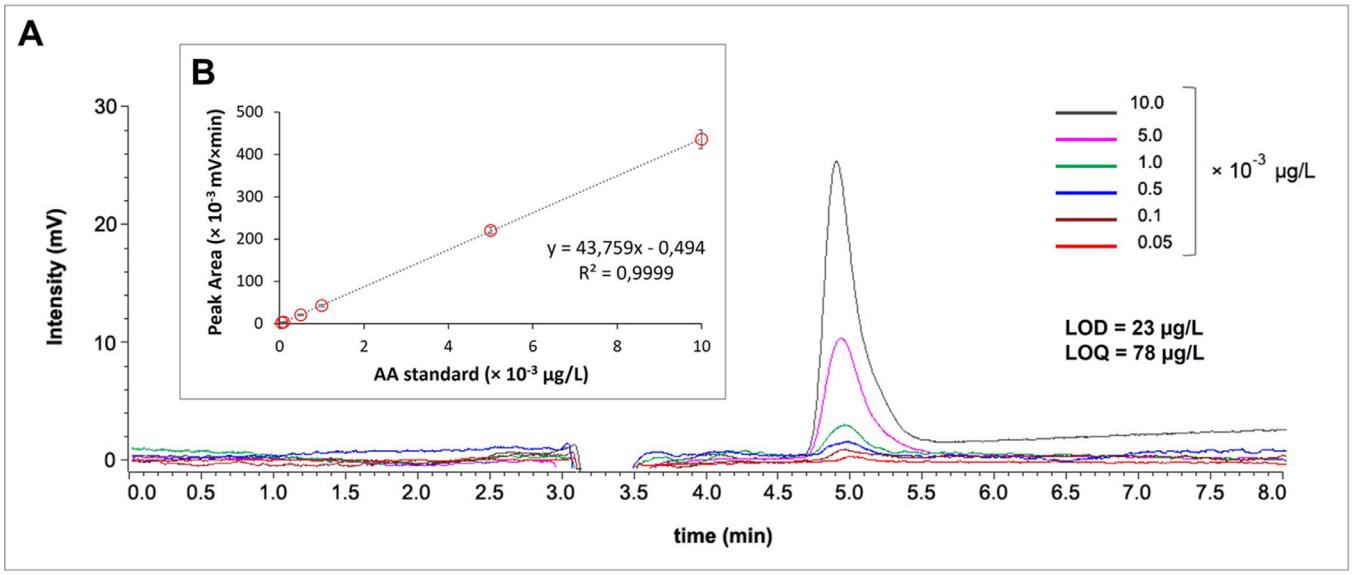

Fig. 1. Chromatograms of Standard AA at different concentrations (50, 100, 500, 1000, 5000 and 10,000 µg/L) by UHPLC-PDA (Jasco, Japan) (A) and calibration curve obtained from the peak area (B). LOD (23 µg/L) and LOQ (78 µg/L) values were calculated from the standard deviation of the lowest AA standard (50 µg/L).

used for dough preparation following the Neapolitan pizza TSG recipe (EU Reg, 97/2010). Briefly, 250 g flour were kneaded together with 61% water, 2.8% salt, 0.17% dry yeast (on the flour weight basis) in the previously described conditions. The proofing was carried out at 22 °C and 80% RH for 8 h, after which the doughs were rolled out until a circular-shaped disc with a central thickness of about 2–3 mm and a higher border (~ 10 mm) was formed. The obtained pizza bases were baked for 1 min at 485±30 °C directly placed on the floor of a handcrafted wood oven for pizzeria, made of refractory bricks, equipped with an internal chamber with a circular base (diameter 90 cm) and a vaulted roof with a maximum central height of 40 cm.

During baking, the temperature of the wood oven was monitored with a thermal imaging camera (FLIR E95 42, FLIR System OU, Estonia) and the position of the pizza was rotated with respect to the floor of the oven, following the specification of the Neapolitan pizza TSG. A wood oven baked pizza base is shown in Figure S2.2 in supplementary material section S2.

2.6. Processing of samples

The obtained pizza samples were cut into pieces for subsequent experiments. The processing of pizza pieces was carried out according to Kumar et al. (2014), with some modifications. The fried pizza samples were preliminarily defatted by shaking in hexane (1:3 w/v) for 30 min at 25 °C and 170 rpm in an orbital shaker (Forma Scientific). After the evaporation of hexane, the samples were frozen, and then, together with the previously frozen wood oven baked pizza pieces, were lyophilized and finely ground at 40-mesh with a coffee grinder. The obtained powder was used for the following experiments of AA extraction.

2.7. Acrylamide standard preparation

A standard stock solution (1.0 mg/mL) was prepared by dissolving 50.0 mg of the acrylamide standard in 50 mL of HPLC water by using a volumetric flask. From the stock solution, calibration standards at different concentrations (50, 100, 500, 1000, 5000 and 1000 µg/L), were prepared, respectively. All series of standard solutions were stored in glass dark bottles (light-resistant) at 4 °C until using. Values reported were averages of three determinations.

2.8. Acrylamide extraction

Two parallel tubes were set up for each sample, one was used as sample test tube, and the second one as recovery test tube in the AA extraction experiments. One gram (on dry weight basis) of freeze-dried pizza powder was placed inside each tube and was defatted with 5 mL of hexane in the same conditions as above described (Kumar et al., 2014). AA from defatted samples was then extracted in water following the protocols reported by Wang et al. (2013) and Al-Asmar et al., 2019, with some modifications. Briefly, AA standard at a final concentration of 5000 µg/L was absorbed on pizza defatted powder in the recovery test tubes, and then 10 mL of HPLC water were added in each tube. The samples were incubated at 25 °C and 170 rpm in an orbital shaker for 30 min, then centrifuged at 8000×*g* for 10 min at 4 °C to allow precipitation of solids. The extraction was repeated twice, and both the supernatant were filtered consecutively through 0.45 and 0.20 μm cellulose acetate filters and stored in refrigerated conditions at 4 °C until UHPLC-UV analyses.

2.9. Acrylamide content determination

The AA content of the pizza samples was determined by using UHPLC (Jasco, Japan) equipped with a reverse phase C-18 column (Nucleodur C18 Gravity 150×3 mm, 3 µm particle size). According to Capuano et al. (2009), Wang et al. (2013), and Kumar et al. (2014), with some modifications, the chromatographic separation was performed at 30 °C using Milli-Q water (solvent A) and Methanol (solvent B), both containing 0.1% formic acid, as mobile phases. The following elution program was applied: 0–3 min 0% B, 4–8 min from 0 to 7% B, 9–12 min from 7 to 100% B (Capuano et al., 2009) at a flow rate of 0.25 mL/min. Absorbance was monitored at 210 nm (PDA detector MD-4010, Jasco). The retention time of authentic AA standard was 4.9 min under given conditions (Fig. 1-A). Peak areas generated by 10 µL of standard AA at different concentrations (50, 100, 500, 1000, 5000 and 10,000 µg/L) were used to build the calibration curve (Fig. 1-B) from which the AA concentration of pizza samples was extrapolated. All analyses were performed in triplicate, and the average results are expressed as $\mu g/kg_{dw}$ (dry weight) of sample. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the standard deviation of the lowest AA standard (50 µg/L) (Magnusson & Örnemark, 2014) and resulted 23 µg/L, and 78 µg/L, respectively.

Table 1
AA content and Recovery test in fried pizza bases untreated (ctr) and treated with several increasing amount of Preventase® W, M and XR-BG.

sample	Preventase® (U/100 g of flour)	AA ($\mu g/kg_{dw}$)	Recovery (%)
ctr	0	3150 ± 554^{a}	99.8
W	0.15	1928 ± 109^{bA}	98.7
	0.3	1577 ± 3^{cA}	95.2
	1.5	1462 ± 66^{cA}	92.2
	3.0	1295 ± 9^{dA}	96.4
	6.0	1129 ± 65^{deA}	93.3
	9.0	383 ± 184^{eA}	77.3
	12.0	n.d.	n.d.
M	0.15	1632 ± 194^{bB}	99.0
	0.3	1611 ± 187^{bA}	98.9
	1.5	1440 ± 130^{bcA}	92.9
	3.0	1227 ± 58^{cA}	98.4
	6.0	801 ± 85^{dB}	94.2
	9.0	374 ± 228^{eA}	89.1
	12.0	n.d.	n.d.
XR-BG	0.15	2054 ± 142^{bA}	97.5
	0.3	2032 ± 141^{bB}	96.1
	1.5	1857 ± 131^{bB}	96.9
	3.0	1640 ± 119^{bcB}	96.2
	6.0	1204 ± 95^{cdA}	95.7
	9.0	768 ± 71^{deB}	93.6
	12.0	332 ± 47^{e}	95.9

Data represent the mean±SD of three replicates (n = 3); nd, not detectable.
Letters indicate samples significance calculated with ANOVA statistical test with Post Hoc Tukey (p<0.05).
Different lower case letters indicate significant differences between samples treated with the same enzyme preparation at different U/100 g.
Different capital letters indicate significant differences between different enzyme preparations used at the same U/100 g.

Percentage of AA recovery was determined according to Al-Asmar et al. (2018) with the following formula:

$$Recovery\ (\%) = \frac{AA\ (detected\ in\ sample\ spiked\ with\ standard\ AA) - AA\ (sample)}{AA\ (standard\ added)} \times 100$$

2.10. Statistical analysis

All experiments and analytical measurements were run in triplicate and the data were expressed as mean ± Standard Deviation (SD). Means of each parameter were evaluated by analysis of variance (ANOVA) using Post Hoc Tukey's test. Statistical analysis was performed using XLSTAT software (version 2014.5.03). Differences between treatments at 5% level ($p < 0.05$) were considered as significant.

3. Results and discussion

3.1. Asparaginase treatment in fried pizza base

The AA content was determined in the untreated (control, ctr) and L-Asparaginase treated fried pizza bases; results are shown in Table 1. The AA level in the ctr sample was 3150±554 $\mu g/kg_{dw}$ and represented the highest value registered for all the analysed samples. Indeed, the AA content in the treated fried pizza bases decreased with increase of enzymatic units for all the types of commercial Preventase®. Specifically, the AA levels in the enzyme-treated samples were in the range of 1928±109 ÷ 383±184, 1632±194 ÷ 374±228, and 2054±142 ÷ 332±47 $\mu g/kg_{dw}$ for W, M and XR-BG respectively (Table 1), with the difference that the Preventase® W and M preparations, used at the maximum concentration of 12 U/100 g, were able to bring the AA to undetectable levels (n.d.), while the XR-BG preparation, at the same quantity, still allowed an AA residue of 332±47 $\mu g/kg_{dw}$.

Analyzing the data numbers in Table 1, it was observed that the estimate of the AA value for Preventase® XR-BG 12 U/100 g was not perfectly accurate, as the concentration of analyte in the extract used for the determination was 33.2 ± 4.7 µg/L, and therefore lower than LOQ (78 µg/L), but still detectable because it is higher than LOD (23 µg/L). Furthermore, the estimates of the M-6 U/100 g and XR-BG-9 U/100 g samples were also very close to the LOQ threshold. Instead, the minimum quantities of AA measured in the W and M samples at 9 U/100 g, are certainly lower than the LOQ but are even at the border of the LOD threshold, considering their high standard deviation.

To confirm the validity of the AA determinations, the extraction efficiency was evaluated by calculating the recovery of the analyte conducted in the presence of 5000 µg/L of AA standard. Analyzing the recovery percentage of AA, it was observed that for most of the samples the value was between 92.2 and 99.0%, and only for the samples prepared with 9 U/100 g of W and M enzyme preparations, the recovery% drops to values of 77.3 and 89.1% respectively (Table 1). However, these are also two of the samples with the lowest AA levels and the highest standard deviation. Probably, at such low levels of AA the matrix effect in the extraction efficiency was more pronounced. However, despite the low accuracy of AA level estimates for samples with high enzyme concentrations, this does not mean that the differences with other samples are not significant. Definitely, for all the samples in Table 1, it can be said that the determined AA values are statistically compatible with the experimental variations.

The enzymatic treatment was, thus, effective in reducing AA formation, and the performance was relatively consistent. The percentage of AA reduction with respect to the control, with increasing enzyme units used in the dough, is shown in Fig. 2-A. The results showed that the XR-BG enzymatic preparation causes a lower percentage reduction of AA at all concentrations used, reaching a maximum reduction of 89% respect to the control without enzyme. On the other hand, W and M preparations exhibited a very similar trend and were able to completely break down the formation of AA in samples with 12 U/100 g (Fig. 2-A). This result is somewhat controversial, given that the same units of enzyme activity were used for the three available enzyme preparations. However, it must be considered that the assays to determine the specific activity of the enzymes were carried out in aqueous solution, with the enzyme and the substrate (free asparagine) dissolved in the reaction buffer, therefore in optimal conditions for the functioning of the asparaginase. In the case of application in the dough, the reaction conditions are certainly different, but the only change in the recipe is the addition of a different enzyme preparation. To justify the different performance of Preventase® XR-BG, it could be assumed a lower mobility of the enzyme in the matrix of the dough due to the presence, in the commercial preparation, of other components which can interfere with the dispersibility of the enzyme in the dough during mixing. Alternatively, there may be other elements present that may act as inhibitors of the enzyme when released into the matrix, compared to the other two preparations. However, these remain only hypotheses since, unfortunately, the composition of these preparations is covered by a patent, and the only information available on the three commercial forms is that, in addition to the enzyme, the powder also contains wheat flour in the case of Preventase® W. and maltodextrins in the case of Preventase® M and XR-BG.

In all cases, Preventase® treatment was able to sensibly reduce AA content in all kinds of fried pizza base samples. The effectiveness of the Preventase® in reducing the formation of AA in other fried products has also been described by other authors: Rottmann et al. (2021) reported an AA reduction of about 60% in pre-treated potato chips with Preventase®. In general, according to our results, the asparaginase enzyme has been proposed as an AA mitigation strategy in different types of products. Vass et al. (2004) experienced already that the addition of asparaginase in two different crackers was able to decrease the amount

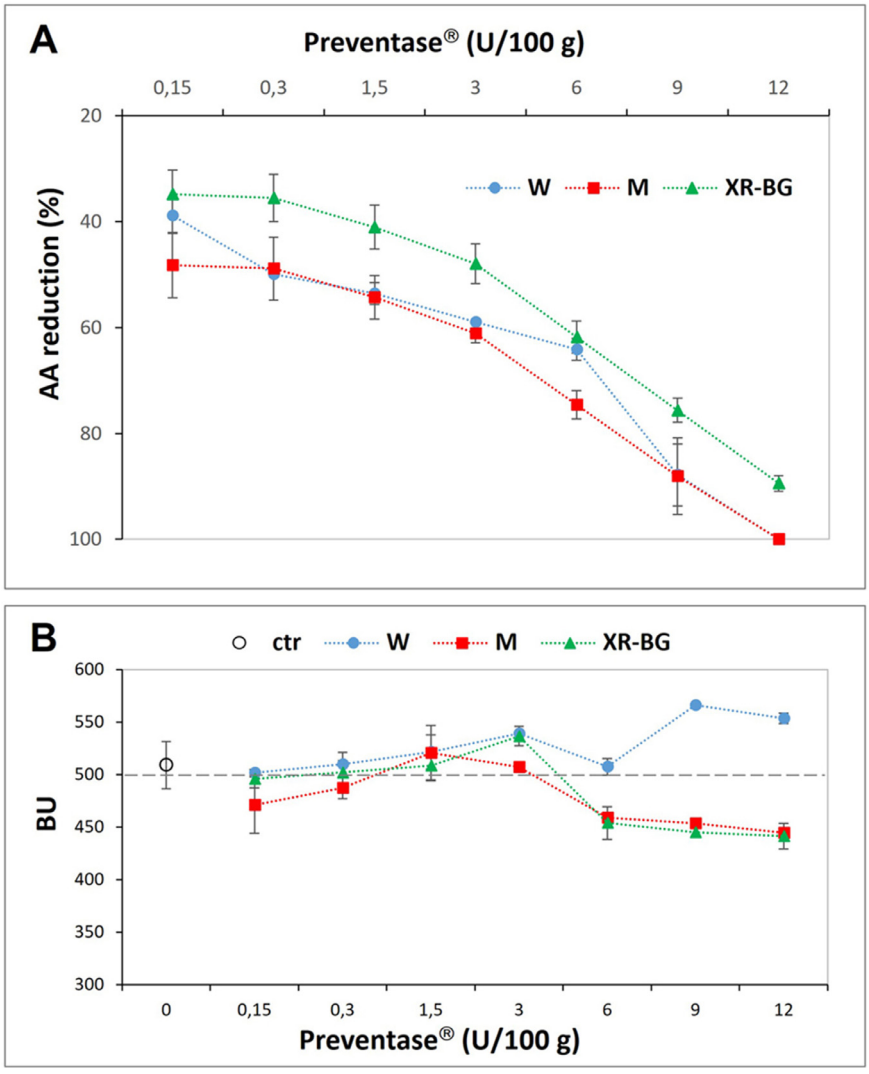

Fig. 2. AA reduction respect to the untreated control sample (ctr) (A) and Brabender Units values (B) of fried pizza bases treated with several increasing amount of Preventase® W, M or XR-BG. Values are expressed as mean±SD of three replicates (n = 3).

of AA formed by 70%. Capuano et al. (2009) found a reduction of AA content in the range of 70 – 88% in bread crisps baked at 160 °C and 180 °C.

3.1.1. Impact of the addiction of the enzyme preparation in the dough on its technological properties

The extent of the AA reduction is not the only factor to be taken into account for choosing the optimal amount of enzymatic preparation to be added to the product in the formulation phase. It is also necessary to consider whether the addition of a technological adjuvant, with its own composition, does not alter the physico-chemical properties of the product. In fact, it is important not to affect the sensory attributes and overall acceptability by consumers. As regards to the kneaded products, a very important parameter for the quality of the dough is the Brabender Units value (BU), determined by the farinograph, which represents a measure of the rheological characteristics of the dough. For kneaded products, such as bread and pizza, an optimal dough should reach 500 BU (Rosell et al., 2001). The replacement of wheat flour with the enzymatic preparation can lead to the variation of the BU, and, consequently, to the modification of the viscosity of the dough. This feature is a very important parameter for the manipulability of the pizza dough which can, subsequently, affect its extensibility and elasticity. Fig. 2-B shows BU values measured for doughs of fried pizza, prepared with the different amounts of enzymes using a constant water level adjusted for reaching 500 BU in the control dough. As can be seen in the figure, at high concentrations of enzyme significant deviations from the threshold of 500 BU are observed for all enzymatic preparations, while BU are acceptable up to 3 U/100 g of flour for preparations M and XR-BG, and up to 6 U/100 g for Preventase® W type. In particular, at 3 U/100 g of Preventase®, the average reduction of AA for the three enzymes is around 50–60%, with the best performance for the M and W enzymes.

3.1.2. Effect of water content on BU of doughs and AA reduction in pizza base

This amount of Preventase® was chosen for subsequent experiments in which the fried pizza base samples were prepared at higher hydration degree (60, 62 and 64 g of water added to 100 g of flour, compared to 58 g of the control). From the literature it is known that the water content of the products, during cooking, can influence the formation of AA (Zyzak et al., 2003; Masatcioglu et al., 2014). In fact, as soon as the dough is placed in the hot oil or in the oven, water evaporates very fast from the surface layers, resulting in much lower water content than at the core. As the water content decreases in the external part of the pizza, the temperature can exceed 100 °C, which supports reactions such a caramelization, carbonization and Maillard reaction, responsible for the browning coloration (Gökmen & Palazoğlu, 2008; Dessev et al., 2020). These reactions belong to the non-enzymatic or non-oxidative browning category. The Maillard reaction is the main responsible for color development at temperatures below 150 °C. Caramelization and carbonization reactions take place at temperatures above 150 °C. The Maillard re-

Table 2
AA content and Recovery test in fried pizza bases, untreated (ctr) and treated with 3 U/100 g of Preventase® W, M, and XR-BG, prepared at different hydration degree.

Sample	Water (g/100 g of flour)	AA ($\mu g/kg_{dw}$)	Recovery (%)
ctr	58	3150 ± 554^{aA}	99.8
	60	2641 ± 119^{aA}	96.9
	62	2190 ± 94^{bA}	97.8
	64	1779 ± 15^{cA}	98.6
W	58	1295 ± 9^{aB}	96.4
	60	1334 ± 130^{aB}	99.5
	62	951 ± 20^{bB}	100.3
	64	306 ± 52^{cB}	101.6
M	58	1227 ± 58^{aB}	98.4
	60	1226 ± 136^{aB}	99.8
	62	987 ± 9^{bB}	100.3
	64	516 ± 45^{cC}	101.2
XR-BG	58	1640 ± 119^{aC}	96.2
	60	1329 ± 77^{bB}	99.6
	62	1067 ± 172^{bB}	100.1
	64	562 ± 257^{cBC}	101.1

Data represent the mean±SD of three replicates ($n = 3$).
Letters indicate samples significance calculated with ANOVA statistical test with Post Hoc Tukey ($p<0.05$).
Different lower case letters indicate significant differences between samples treated with the same enzyme at different% of water. Different capital letters indicate significant differences between samples treated with different enzymes at the same% of water.

action is influenced by composition in reducing sugar and amino acids, but also by temperature, pH and water content (Zanoni et al., 1995). The water content seems to play an important role in any such degradation process: acrylamide content decreases simultaneously with water content increasing (Sadd et al., 2008; Masatcioglu et al., 2014). This phenomenon is explained by considering that the dehydration phase is a crucial step in the formation of AA; the increase in water content represents a limitation with consequent inhibition of the reaction (Zyzak et al., 2003).

To investigate the combined effect of the asparaginase in pizza bases with a higher hydration degree, doughs with a water content increasing from 58 g/100 g of flour (used in the previous recipe) to 64 g/100 g of flour were prepared, keeping the Preventase® concentration fixed at 3 U/100 g. The content of AA determined in these samples is shown in Table 2. All measured values are above the LOQ except those with water content of 64 g/100 g of flour for all enzymatic preparations used, which, however, do not fall below the LOD threshold. A lower concentration of AA is confirmed in the enzymatically treated pizza bases (W, M and XR-BG) compared to the untreated controls (ctr), at all the considered hydration percentages. Moreover, acrylamide levels measured for each typology of pizza base decreased with increasing of water content in the corresponding doughs, with significant differences especially at hydration levels equal to or greater than 62 g/100 g of flour (Table 2).

The increase in the water content of the dough has proved to be a very effective method to reduce the AA in fried pizza bases, in fact in the untreated sample there was a reduction of 16, 30 and 44 percentage points passing from 60 to 64 g/100 g of flour of hydration compared to the 58 g/100 g of flour. In enzymatically treated samples, the performance of the 3 enzymatic preparations was practically comparable from 60 g/100 g of flour onwards, and the extent of the reduction even exceeds 80% with only 3 U/100 g of enzyme, at the maximum hydration considered (Fig. 3-A). Such a percentage of reduction, in samples at 58 g/100 g of flour hydration, can only be achieved by using an amount of enzyme at least 3 times higher (Fig. 2-A).

Other authors have reported a reduction in AA levels in products with higher moisture. In a study published in the 2007, Ahrné and, coll. measured the AA content in the bread crust and showed that the amount of AA in the inner crust fraction (the one close to the crumb that has a water content between 6 and 10%) was 25–75% of that in the outer fraction (containing 2–4% of water). More recently, Masatcioglu et al. (2014) achieved a reduction of 54 and 85% in corn extrusions by increasing the feed moisture from 22 to 24 and 26%, respectively.

The obtained results agree with the literature, but also highlight the absence of a synergistic effect of the water content and asparaginase activity on the inhibition of the AA formation reaction. In fact, the reduction curves of AA in the absence and presence of Preventase® seem to proceed in parallel. In this experiment, for the control sample, the AA reduction rate has practically a linear trend with an $R^2 = 1$ (Figure S3.1-D). Even in the samples containing 3 U/100 g of Preventase® the reduction rate shows a certain linearity, that extends for the whole considered range for the XR-BG samples (Figure S3.1-C) and the samples W and M with a hydration degree from 60 to 64 g/100 g of flour (Figure S3.1-A and B). The slopes of these lines fall within a very narrow interval (11 ÷ 16 percentage points), suggesting a parallel trend of the AA reduction phenomena. In the presence of synergistic phenomena, a greater slope of the lines corresponding to the samples treated with the enzyme, compared to that of the control, would have been expected. However, this did not occur, indicating that, in all the samples, the increase in the reduction of AA is essentially due to the higher percentage of water present (Fig. 3-A).

Although it has proved to be an effective AA mitigation strategy, increasing the percentage of water in the doughs is not a viable formulation change for pizza dough processing, as hydration alters the technological parameters of the doughs, starting with viscosity. As a demonstration of this, a collapse of the BUs from 500 to about 300–350 is observed for all the samples passing from 58 to 64 g/100 g of flour hydration (Fig. 3-B). This means that the dough loses consistency and becomes more sticky and difficult to handle.

Thus, in view of an intervention for the reduction of the AA in fried pizzas, in order not to affect the technological characteristics of the dough, it is preferable to resort to the use of asparaginase, especially the Preventase® W for which up to 6 U/100 g of flour can be used without significantly altering the BU compared to the control (Fig. 2-B).

3.2. AA reduction in wood oven baked pizza base

The action of asparaginase in reducing AA was also evaluated in pizza base samples cooked in a wood oven. In this experiment, 1.5, 3 and 6 U/100 g of Preventase® W were used in the preparation of pizza dough. These quantities were chosen on the basis of previous experiments, because they proved to be effective on average in bringing the reduction of AA to values higher than 50% in the fried pizza samples (Fig. 3-A). Furthermore, Preventase® W has been selected among the three, because it contains wheat flour in its composition, and therefore its use does not involve the addition of other ingredients to the recipe, while the other two preparations contain maltodextrins that can interfere with the methods and times of dough development.

Results of the pizza base AA content and reduction, and the BU of the corresponding doughs are shown in Fig. 4. All measured AA values were higher then LOQ, but unfortunately the recovery values were between 88.0 and 90.2%, and therefore significantly lower than those of fried pizza bases, which for the same enzyme units were greater than 92.2%. Anyway, compared to the fried untreated sample, the pizza base cooked in a wood oven showed a lower AA level (2210 ± 205 *vs* 3150 ± 554 $\mu g/kg_{dw}$). This is not surprising, as it is known that frying processes are characterized by higher levels of Maillard reaction with all the consequences, compared to oven baking (including the wood oven) (Danowska-Oziewicz et al., 2007; Zhang & Zhang, 2008; Friedman & Levin, 2008). Moreover, it must also be considered that the doughs for the pizza base baked in the wood oven were produced with a different flour than those of the fried pizza bases, and also the leavening process, before reaching the cooking, was longer (8 h *vs* 2 h) because the recipe of

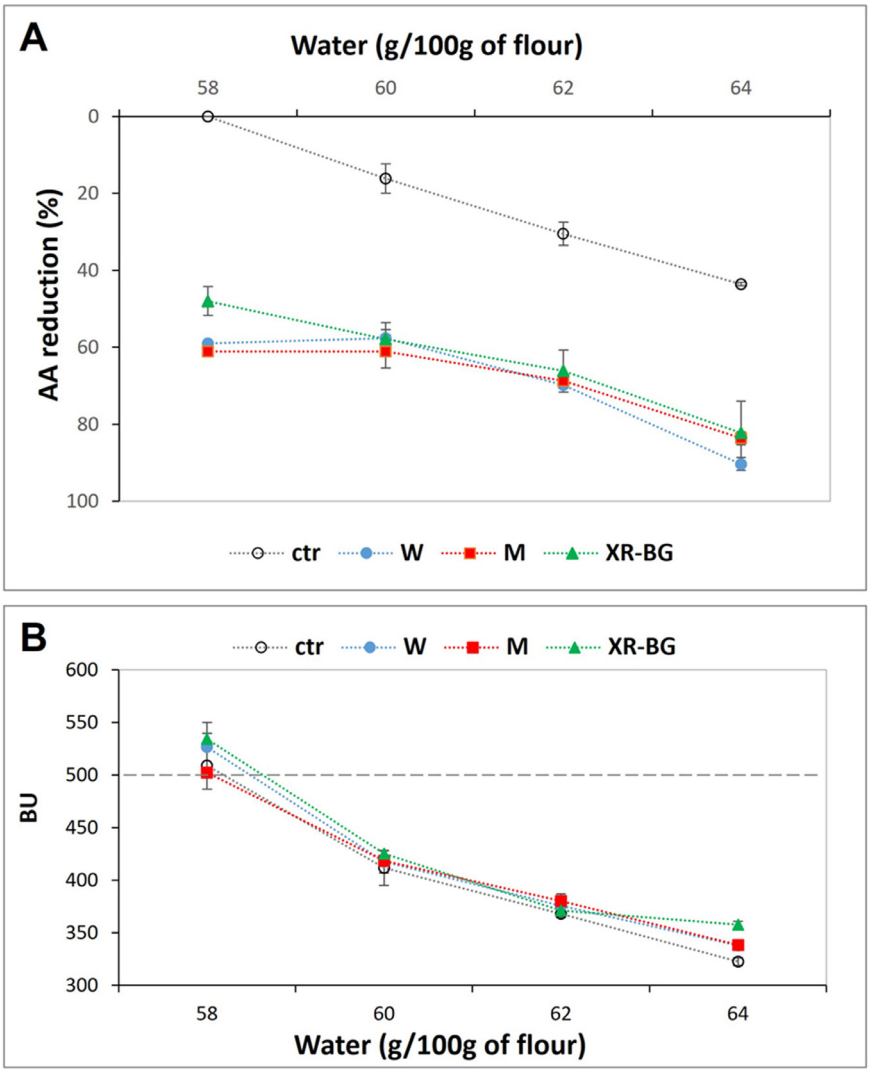

Fig. 3. AA reduction (A) and Brabender Units values (B) of fried pizza base prepared at different hydration degree (58, 60, 62 and 64 g of water/100 g of flour), untreated (ctr) and treated with fixed amount (3 U/100 g) of Preventase® W, M or XR-BG (3 U/100 g). Values are expressed as mean±SD of three replicates (n = 3).

the TSG specification for Neapolitan pizza was followed. Consequently, the amount of reducing sugars and free Asn-in the two different doughs at the moment of cooking could be different both for the different composition of the starting flours and for the different metabolic activity of the yeasts (Fredriksson et al., 2004; Claus et al., 2008; Wang et al., 2017).

However, as observed for the fried pizza bases, also for the wood oven baked ones, the AA levels decreased with increasing amounts of asparaginase in the dough (Fig. 4-A), and the performance of the enzyme seemed to be comparable to that observed in previous experiments, at least at 3 and 6 U/100 g where a maximum of 46–49% of reduction was reached. Only at 1.5 U/100 g there is a lower efficiency of the Preventase® W with a reduction of AA of 12% (*vs* 54%).

As regards the technological aspects of the dough for pizza base TSG, also in this case, the addition of Preventase® W to the ingredients, replacing the homologous quantities of flour, did not lead to an excessive variation of the BU (Fig. 4-B). Based on the obtained results, it can be stated that Preventase® W was effective in the strategies for mitigation of acrylamide also in products cooked with methods other than frying, such as the wood oven baking.

3.3. Safety issues and consumer concerns

Since its discovery in food, the organizations responsible for food safety have been committed to evaluating toxicity studies of acrylamide and supporting information campaigns to increase consumer awareness. For European countries, EFSA has established a limit threshold for the daily intake of AA equal to 170 μg/kg of body weight (EFSA, 2015:EN-817). This means that an average person weighing 70 kg must not exceed a maximum dose of AA of 11,900 μg in one day.

From the results reported in this study, it can be deduced that for a pizza base of 250 g, considering a dry substance of 65%, the AA content is equal to about 512 μg for a fried pizza base, and about 360 μg for a base pizza cooked in a wood oven. In both cases, the values are far below the threshold established for an individual weighing 70 kg, and precisely the dose limit/dose taken ratio is 23 and 33 respectively. There is a further consideration to be made: fried pizza is seasoned after cooking, therefore the entire surface of the product is exposed to the formation of AA; on the contrary, for pizza cooked in a wood oven, the AA content can even drop below the value of 360 μg calculated for the pizza base. In fact, wood oven baked pizza is generally cooked in the presence of topping that covers the upper surface of the dough disc, and this reduces the exposure to the Maillard reaction except on the pizza rim. Thus, the AA is essentially distributed on bottom and the rim surfaces of the pizza.

Considering that a 250 g dough ball produces a circular pizza with an average diameter of 27±1 cm, the total surface area of the pizza base can be calculated as the area of two circles 2 (bottom disc + upper disc) and corresponds to 2 × 573±60 cm^2 (about 1146 cm^2) (Figure S4.1). Therefore, in a pizza base baked in a wood oven, a distribution of AA equal to 0.31 μg/cm^2 can be deduced. Since in a pizza garnished

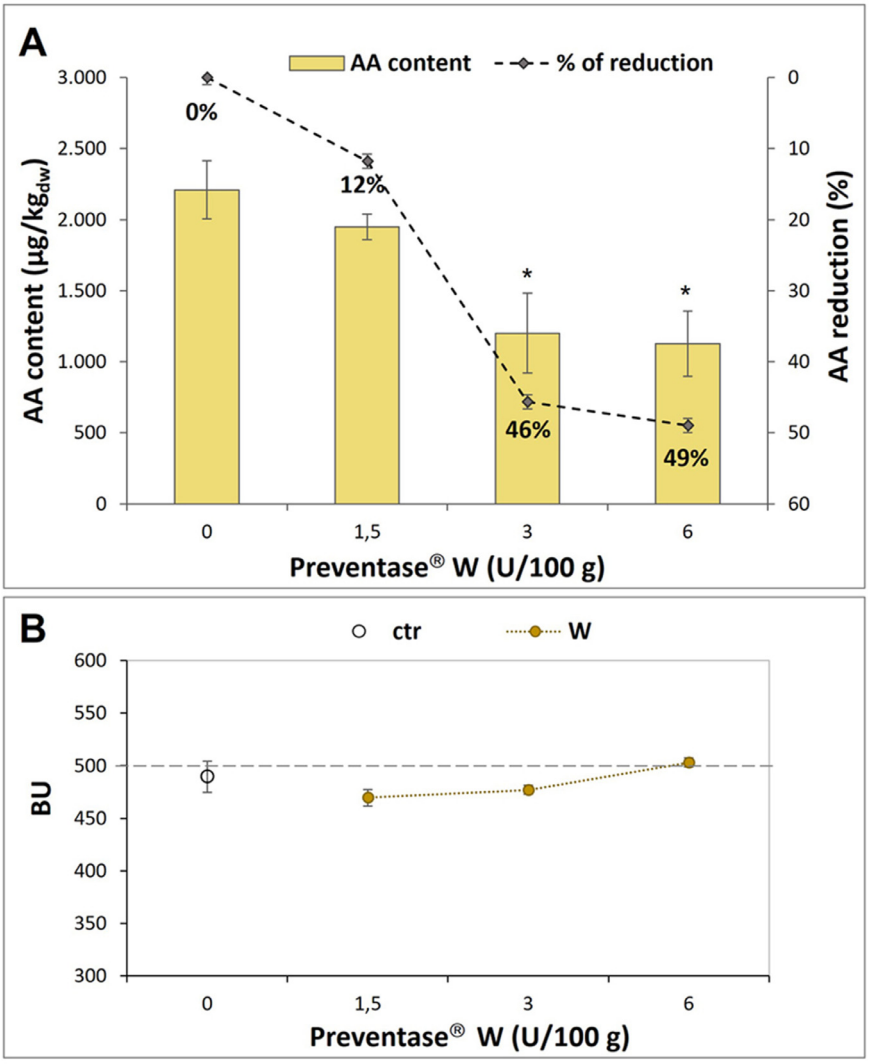

Fig. 4. AA content and reduction (A) and Brabender Units values (B) of wood oven baked pizza base treated with 1.5, 3 and 6 U/100 g of Preventase® W. Values are expressed as mean±SD of three replicates (n = 3).

with sauce, the upper disck is exposed to the formation of AA only in the part of the rim, which for a Neapolitan pizza is about 2 cm (Reg. EU, 2010), eliminating the surface of the central disc with a diameter of 23±1 cm (416±51 cm^2), it can be calculated that the pizza surface affected by the presence of AA is about 730±69 cm^2 (Figure S4.1), and that the AA content is reduced to about 226±21 µg. For this value, the dose limit/dose taken ratio is around 53. Finally, it is noteworthy that in a real case of pizza baking, even the presence of the topping can affect the AA formation, by slowing down the increase of pizza temperature.

The use of 3 U/g of Preventase® W can lead to a reduction of about 60% and 40% of AA in the fried and wood oven baked pizza bases respectively. Consequently, the intake of AA also drops to about 204 µg and 136 µg, with a dose limit/dose taken ratio of 58 and 87.

In any case, it should be remembered that AA is contained in many foods that are consumed daily such as bread, biscuits, crackers, french fries etc. and this is the reason why EFSA encourages the food industry to develop processes that can reduce the AA content in food as much as possible.

4. Conclusions

To our knowledge, this is the first time that the asparaginase enzyme has been applied in the production of pizza dough, especially in the Neapolitan style TSG. The experimental results reported in this study demonstrated its effectiveness. In particular, the most performing preparation was Preventase® W which, with 3 U/100 g of flour, allowed to obtain a maximum reduction of AA of 61% in fried pizza bases and 46% in those cooked in a wood oven. In this way, for an individual of 70 kg the calculated dose limit/dose taken ratios, for fried and wood oven baked pizza, pass from 23 to 52 to 58 and 87 respectively. Moreover, at this enzyme concentration, there were no significant deviations from the threshold of 500 BU in the produced doughs, indicating a low interference of the technological adjuvant on the rheological properties of the product. Due to the wheat flour based composition of Preventase® W, this enzymatic preparation is particularly suitable for direct addition to the flour intended for the production of pizza dough. However, other aspects regarding the rheological properties of doughs and the physico-sensory characteristics of cooked pizza need to be investigated.

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Conflicts of Interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Clelia Covino: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Angela Sorrentino:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Prospero Di Pierro:** Methodology, Validation, Formal analysis, Supervision, Project administration. **Alessandra Aiello:** Methodology, Formal analysis, Investigation. **Raffaele Romano:** Methodology, Formal analysis. **Paolo Masi:** Supervision, Project administration, Funding acquisition.

Data Availability

No data was used for the research described in the article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2023.100206.

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